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Characterization of Three Plasmid Deoxyribonucleic Acid Molecules in a Strain of *Streptococcus faecalis*: Identification of a Plasmid Determining Erythromycin Resistance

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Three plasmids designated α, β, and γ, distinguishable by their molecular weights (6, 17, and 34 million, respectively) were isolated from *Streptococcus faecalis* strain DS-5 (ATCC 14508). Derivatives of this strain "cured" for erythromycin resistance lacked the β-plasmid. In the parent strain the β-plasmid was estimated to be present to the extent of one to two copies per chromosomal genome equivalent whereas the α- and γ-plasmids were about nine and five copies, respectively.

The frequency of appearance of drug-resistant isolates among Lancefield group D streptococci (enterococci) has been increasing steadily in recent years (10). Even though a number of drug-resistance determinants in the genus *Staphylococcus* and the family *Enterobacteriaceae* have long been known to reside on plasmid DNA molecules, the existence of plasmids in streptococcal species has only recently been reported (5, 7). In this investigation we present data on the isolation and characterization of three different plasmid deoxyribonucleic acids (DNA) in a strain of *Streptococcus faecalis* (a member of the group D streptococci) and demonstrate that one of these plasmids is associated with resistance to erythromycin and lincomycin.

MATERIALS AND METHODS

Materials. Reagents and sources were as follows: Sarkosyl NL 30 (sodium dodecyl sarcosinate) from the Geigy Chemical Corp; egg white lysozyme, Pronase, and ethidium bromide from Calbiochem; CsCl (optical grade) from Schwarz BioResearch, Inc.; erythromycin (Ilotycin Gluceptate) from Eli Lilly & Co.; lincomycin (Lincocin) from the Upjohn Co.; acridine orange from Chem Service Inc.; thymidine-*methyl*-^{3}H (22 Ci/mmol from Amersham/Searle; and thymidine-*methyl*-^{14}C (51.5 mCi/mmol) from New England Nuclear Corp.

Bacteria and media. *S. faecalis* ATCC strain 14508 (designated here as DS-5) was used throughout this investigation. This strain is resistant to erythromycin (>1 mg/ml), lincomycin (>1 mg/ml), and tetracycline (up to 150 μg/ml). Difco antibiotic medium no. 3 (Penassay broth) was used as a growth medium in both liquid and semisolid (agar) forms. Cell growth was at 37 C and was followed by measuring turbidity in a Klett-Summerson colorimeter.

Curing. Curing experiments were performed by growing cells overnight in the presence of acridine orange (50 μg/ml) or ethidium bromide (2 to 10 μg/ml) in either liquid medium or on plates. After plating on agar (in the absence of the curing agent) colonies were replica-plated onto media containing 25 μg of erythromycin per ml. Each procedure produced 1 to 2% curing of erythromycin resistance, whereas spontaneous curing was not observed at a frequency of greater than 0.1%.

Preparation of Sarkosyl lysates. Cells were harvested and resuspended in 1 ml of 25% sucrose (0.05 M tris[hydroxymethyl]aminomethane [Tris], pH 8.0). Lysozyme was added (0.2 ml of 5 mg/ml solution in TES [0.03 M Tris-0.005 M Na_2 ethylenediamine tetraacetic acid [EDTA]-0.05 M NaCl] pH 8.0), and after 5 min at 25 C, 0.4 ml of 0.25 M EDTA, pH 8.0, was added. After another 5 min at 25 C, Pronase was added (0.2 ml of a 5 mg/ml solution in TES buffer, predigested at 37 C for 30 min). After 5 min lysis was brought about by the addition of 1.8 ml of 2% Sarkosyl (in TES).

Centrifugation procedures. Dye-buoyant density centrifugation and sucrose density gradient centrifugation (including the nature and source of the 23S ColE1 marker DNA), fractionation of gradients, and the counting of radioisotope were as described in detail previously (3, 4). For both types of gradients, the recoveries of the applied radioactive material were greater than 90%.

Electron microscopy. Analysis using the basic protein film technique (6) was performed with an Hitachi HS-8 electron microscope. Tracings of open circular molecules were made after projection of negatives on a Nikon profile projector model 6C. From map-tracer measurements, contour lengths were calculated from the known magnification determined

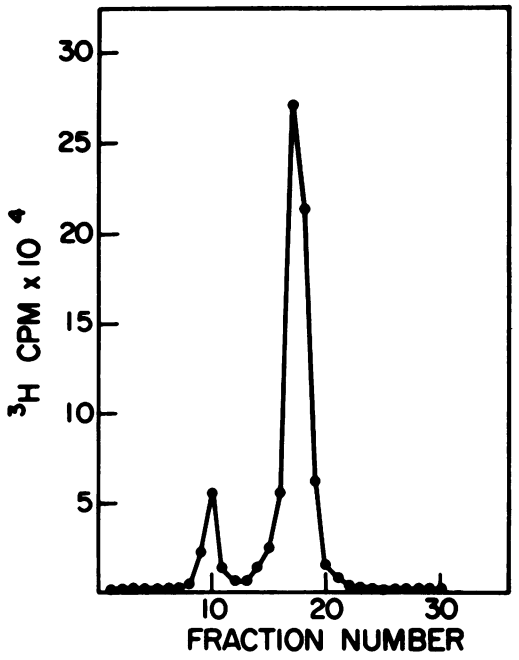

FIG. 1. *Dye-CsCl buoyant density gradient centrifugation of crude lysate of S. faecalis strain DS-5. Log phase cells growing in 15 ml of broth, containing 150 μCi of ^{3}H-thymidine, were harvested and lysed by the Sarkosyl-procedure. A 1-ml amount of lysate was centrifuged to equilibrium (40,000 rpm in a Beckman Ti50 fixed angle rotor for 60 h at 15 C) in a CsCl gradient containing ethidium bromide. The gradient was fractionated and the amount of radioactivity in 0.05-ml samples of each fraction was determined. Density increases from right to left.*

with a calibration grid. Molecular weights were estimated on the assumption that 1.0 μm corresponds to 1.96×10^6 of DNA (8).

RESULTS

Isolation and characterization of covalently closed circular (CCC) DNA. A log-phase culture of *S. faecalis* strain DS-5, grown in the presence of ^{3}H-thymidine, was harvested and lysed by the Sarkosyl procedure. A sample of the lysate was centrifuged to equilibrium in a dye-buoyant density gradient giving rise to the density profile shown in Fig. 1. A "satellite" component representing about 15% of the total DNA is seen at a position of higher density than the bulk (chromosomal) DNA. Such a satellite component, which was not observed in a CsCl buoyant density gradient in the absence of ethidium bromide, is characteristic of CCC DNA. When fractions containing the satellite DNA were pooled, dialyzed, and analyzed on a sucrose density gradient in the presence of a ^{14}C-labeled marker DNA, the profile shown in Fig. 2 was obtained. Figure 2A reveals three peaks corresponding to about 58, 38, and 28*S*. After storage in solution for 2 weeks the same sample gives rise to the profile shown in Fig. 2B where it is seen that the relative amounts of 58 and 38*S* substances have changed. This is a characteristic of the larger supercoiled molecules which, in our hands, tend to undergo spontaneous nicking upon storage in solution (electron microscope analysis of these preparations reveal mixtures of large supercoiled and open circular [nicked] molecules). Smaller molecules such as the 28*S* substance would generally be less likely to acquire a nick, either spontaneously or due to the presence of low levels of nuclease activity. When a sucrose density gradient is run for a longer period of time and is performed very soon after separation in the dye-buoyant density gradient, it is observed (Fig. 2C) that the material previously seen to sediment around 38*S* actually consists of two populations of molecules: one containing a 43*S* DNA and one containing the nicked form (38*S*) of the 58*S* supercoil. The 43*S* DNA is not apparent in preparations that have been stored for several weeks, owing to its lower concentration (relative to increasing amounts of 38*S* material).

Electron microscope analysis of the DNA from the dye-CsCl satellite component has clearly established three size classes of molecules which are readily distinguishable (Fig. 3 and 4A). A number of catenated dimers of the largest size class were also observed. Contour length measurements of open circular molecules indicate molecules of molecular weights of 5.8, 16.9 and 34.3 million. These plasmids have been designated, respectively, α, β, and γ. Their molecular weights on the basis of contour lengths correspond reasonably well with values that can be estimated from the sedimentation rates of the three supercoils (2). In the latter calculation the 43*S* molecule (β) was assumed to be supercoiled. The data is summarized in Table 1 which contains, in addition, estimates of the number of copies of each plasmid per chromosomal genome equivalent (CGE). The latter estimation was based on the molecular weights (from the contour lengths) of each plasmid, the amount of each plasmid taken as a fraction of the chromosomal DNA, and the assumption that the genome size of *S. faecalis* DNA is 1.47×10^9 (1). The α- and γ-plasmids were found to be present in multiple copies, 8.7 and 5.3, respectively, whereas the β-plasmid was present to the extent of 1.5 copies. These are minimum values because plasmid molecules

not initially in a covalently closed configuration are excluded.

Location of the erythromycin-resistance determinant. Curing for erythromycin resistance could be accomplished to the extent of about 1 to 2% by using either of two known curing agents, acridine orange or ethidium bromide. When an inoculum of cured cells ($\sim 5 \times 10^7$ cells) was spread on a plate containing 25 μg of erythromycin per ml, no colonies appeared indicating no readily occurring reversion. All cells cured for erythromycin also became sensitive to lincomycin (20 strains were examined).

Table 2 shows the minimum inhibitory concentrations (MIC) of these two drugs in the parent strain and a cured derivative. Cured cells were still resistant to tetracycline (MIC = 300 μg/ml). Efforts to cure for tetracycline resistance were unsuccessful.

To determine which, if any, of the three plasmids was responsible for erythromycin resistance the following experiment was performed. A cured strain was grown in the presence of ^{14}C-thymidine, while the parent resistant strain was grown in the presence of ^{3}H-thymidine. The two cultures were harvested, mixed, lysed, and centrifuged to equilibrium in a dye-buoyant density gradient. The result shown in Fig. 5 indicates that with regard to the magnitude of the satellite components little difference was observed between the cured and uncured strains. However, when analyzed on a sucrose density gradient (Fig. 6) the ^{14}C counts failed to reveal the 43*S* β-plasmid. An essen-

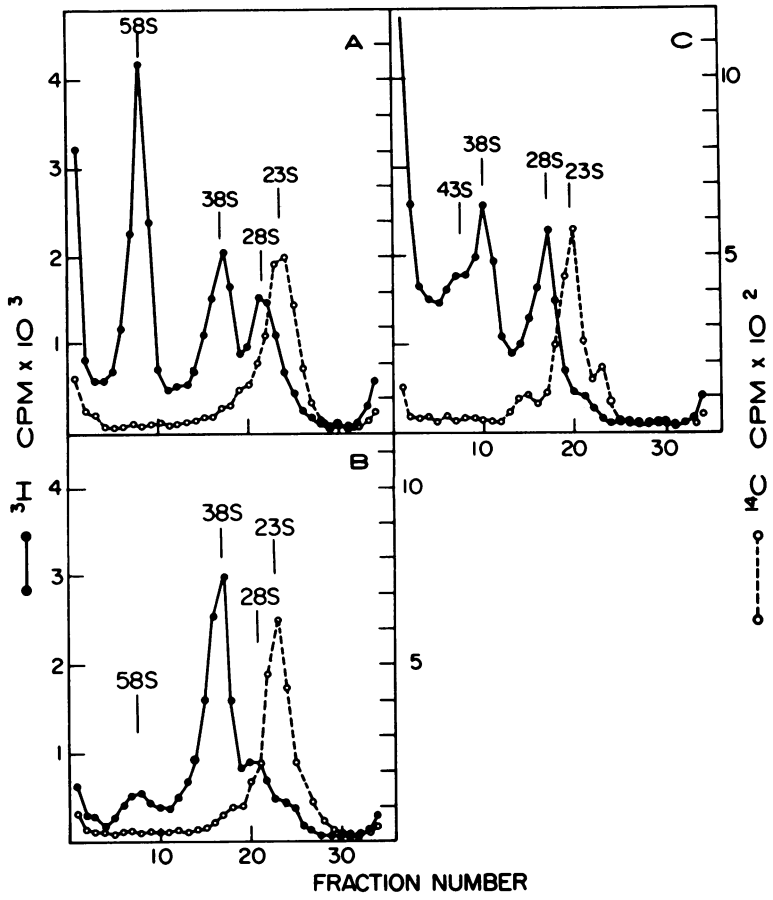

FIG. 2. *Sedimentation analyses of CCC DNA from S. faecalis DS-5. Fractions 9–12 in the gradient of Fig. 1 were dialyzed against 0.015 M NaCl-0.0015 M Na citrate, pH 7.4. ^{14}C-labeled 23S ColE1 DNA, serving as a marker, was mixed with the sample and 0.2 ml was centrifuged (from right to left) through 5 to 20% sucrose density gradients in an SW50.1 rotor (15 C) at 48,000 rpm. A, A 60-min centrifugation of freshly prepared CCC DNA. B, A sample of the same preparation of DNA used in A, analyzed 2 weeks later. C, The same DNA as in A (fresh), except centrifuged for 85 min.*

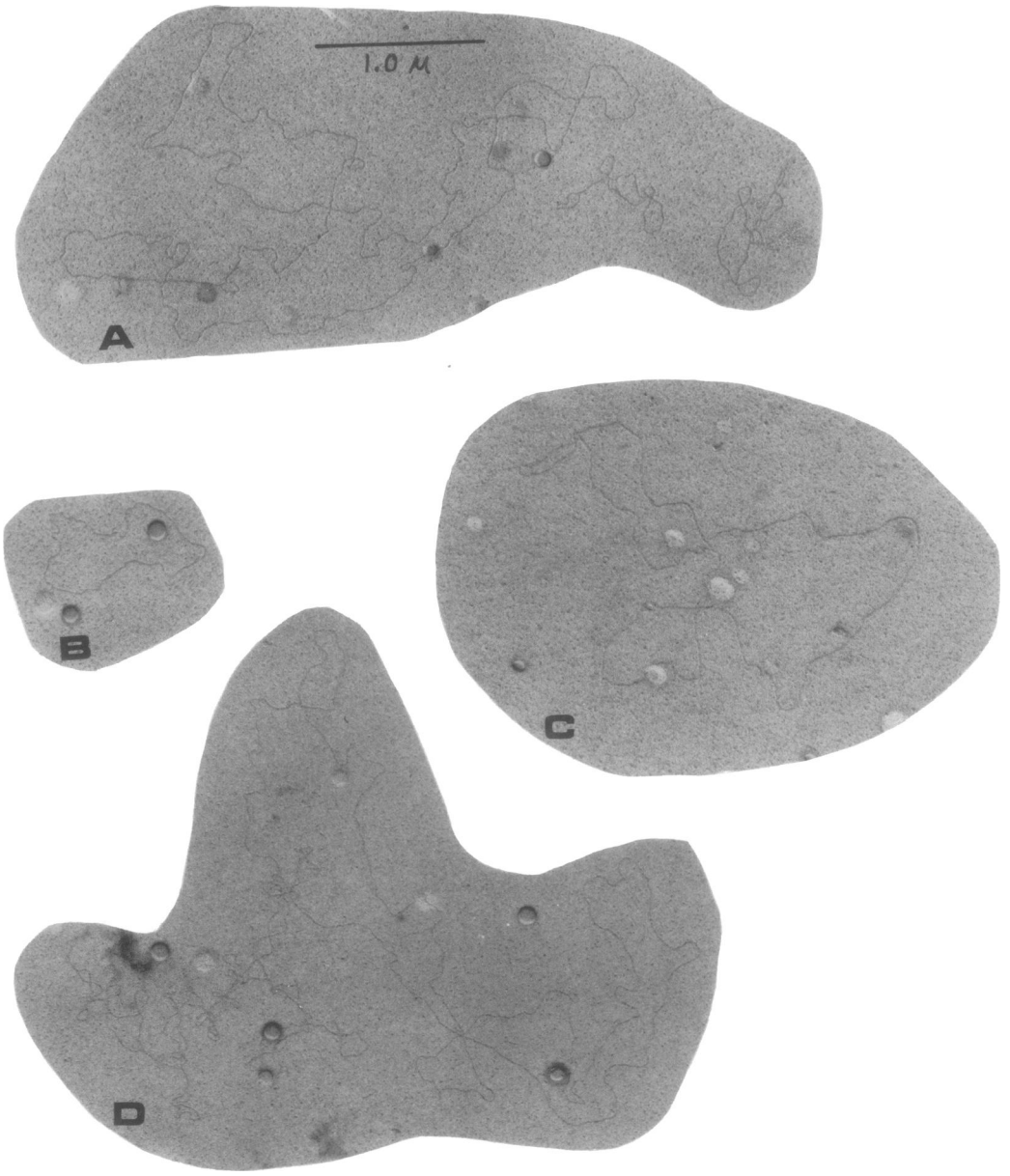

FIG. 3. *Electron micrographs of DNA molecules isolated from S. faecalis DS-5. A, An open circular γ-plasmid with 2 small α-plasmids nearby; B, an open circular α-plasmid; C, an open circular β-plasmid; D, an open circular γ-plasmid that appears to be interlocked with a supercoiled γ-plasmid.*

TABLE 1. *Summary of the physical properties of plasmid DNA molecules from S. faecalis strain DS-5*

Plasmid	Sed. Const. of CCC form	Mol wt $\times 10^{-6}$ based on Sccc[a]	Contour length (μm) $\pm$ standard deviation	Mol wt $\times 10^{-6}$ $\pm$ standard deviation based on contour length	Percent of total plasmid DNA[b]	Percent of chrom. DNA[c]	Avg. no. of copies per chrom. genome equivalent[d]
α	28S	6.5	2.98 $\pm$ 0.22	5.8 $\pm$ 0.43	19.3	3.38	8.7
β	43S	17.7	8.62 $\pm$ 0.38	16.9 $\pm$ 0.74	10.1	1.76	1.5
γ	58S	35.6	17.52 $\pm$ 0.74	34.3 $\pm$ 1.45	70.6	12.35	5.3

[a] The equation Sccc = 0.034 $M^{0.428}$ was used to estimate molecular weight (2). Sccc refers to the sedimentation constant of CCC DNA.

[b] This was estimated on the basis of the relative areas confined by each component in the sucrose density gradient profiles of Fig. 2A and C. The γ-plasmid is represented by the sum of the 58 and 38S peaks and also includes material observed at the bottom of the gradient (likely to be representative, to some extent, of catenated dimers). A second independent estimation (using the ^3H counts of Fig. 6) gave values of 17.4, 12.2, and 70.4% for α, β, and γ, respectively.

[c] These values were based on the assumption that the total plasmid DNA represented 17.5% the level of the chromosomal material in a dye-CsCl gradient. The latter percentage was the average of six independent determinations ranging from 14.5 to 18.3%.

[d] These calculations were based on the assumption that the genome size of *S. faecalis* DNA is 1.47 $\times 10^9$ daltons (1).

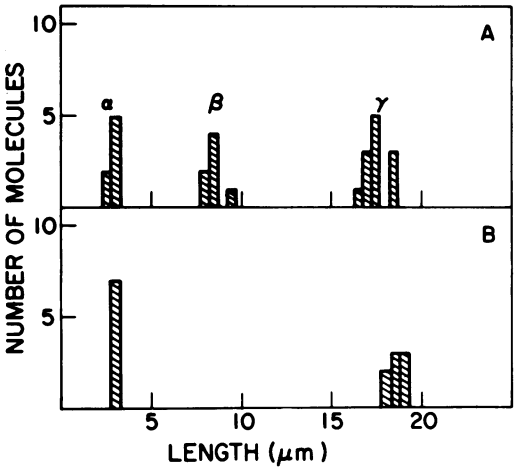

FIG. 4. *Distribution of contour lengths (to the nearest 0.5 μm) of circular molecules of DNA from: A, S. faecalis DS-5; and B, a derivative of strain DS-5, cured of erythromycin resistance. Catenated molecules were ignored.*

TABLE 2. *MIC of erythromycin (Ery) and linocomycin (Linco)*

Strain[a]	MIC (μg/ml)	
	Ery	Linco
DS-5	>1,280	>1,280
DS-5C1	2.5	40.0

[a] DS-5C1 is a strain cured of erythromycin resistance using acridine orange.

tially identical result was found in three different experiments using three independently cured strains. Electron microscope analysis of DNA from one of these cured strains revealed

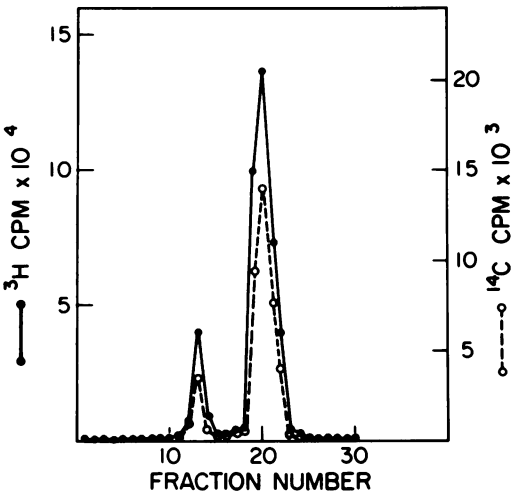

FIG. 5. *Dye-CsCl buoyant density gradient centrifugation of crude lysate consisting of a differentially-labeled mixture of wild-type cells and cells cured of erythromycin-resistance. Chilled cells from a 15-ml log culture of the wild-type strain labeled with ^3H-thymidine (150 μCi) were mixed with a similarly grown culture of ^{14}C-labeled (30 μCi) cured cells. A 1-ml sample of lysate prepared from this mixture was centrifuged to equilibrium (40,000 rpm in a Beckman Ti50 fixed angle rotor for 60 h at 15 C) in a CsCl gradient containing ethidium bromide. The gradient was fractionated and the amount of radioactivity in 0.05-ml samples of each fraction was determined. Density increases from right to left.*

molecules of a size class corresponding to the α- and γ-plasmids (Fig. 4B); however, extensive searching failed to reveal molecules corresponding to the β-plasmid. It is therefore concluded that the determinant(s) for erythromycin resistance in strain DS-5 is borne on the β-plasmid.

DISCUSSION

The data presented here clearly reveal the presence of three plasmids, readily distinguishable by their sizes, in a strain of *S. faecalis* resistant to erythromycin, lincomycin, and tetracycline. Molecular weight estimates based on the sedimentation rates in sucrose density gradients agree well with values based on molecular contour lengths measured by electron microscopy. Designated α, β, and γ, the approximate molecular weights of these plasmids are 6, 17 and 34 million, respectively. Failure to detect the β-plasmid in strains cured for erythromycin resistance strongly implicates this molecule as the carrier of the resistance determinant. Additionally, it was found that cured cells acquire a sensitivity to lincomycin; and in view of previous reports in other systems on the close relationship of resistance to these two drugs (9, 11) it is very possible that resistance to both involve common loci.

Although it is a reasonable possibility that the tetracycline-resistance determinant in this strain resides on one of the other two plasmids, efforts to cure the strain of resistance to this drug have so far been unsuccessful. If the latter determinant is in fact borne on the α- or γ-plasmid, difficulty in curing may arise from the relatively large number of copies of these plasmids (about 9 and 5, respectively, per chromosomal genome equivalent). It is interesting, in this regard, that the easily curable β-plasmid is present to the extent of only one to two copies per CGE. The presence of multiple copies of a plasmid may help provide some insurance against loss by segregation.

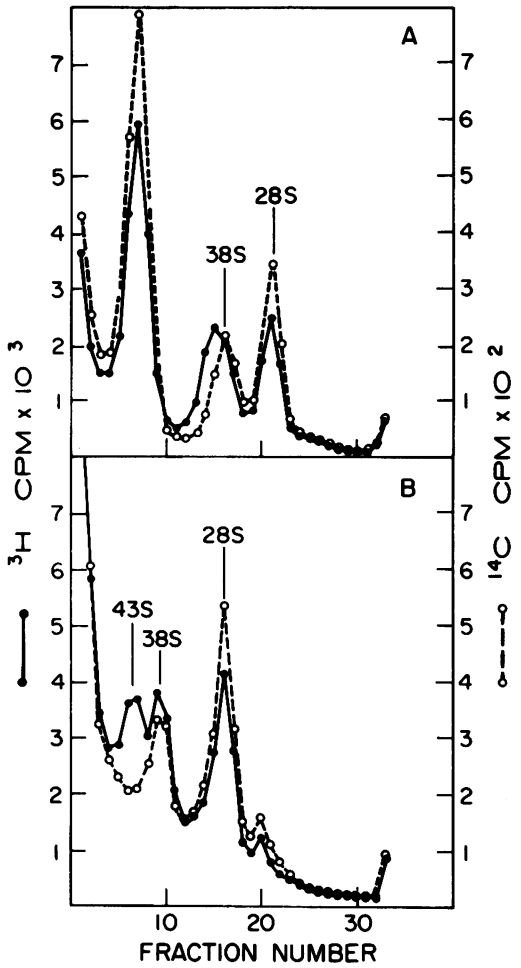

FIG. 6. *Sedimentation analyses of CCC DNA from differentially labeled cured and uncured strains. The sample represents a pool of fractions 12 to 14 in the gradient of Fig. 5. A 0.2-ml sample (previously dialyzed) was centrifuged (from right to left) through 5 to 20% sucrose density gradients in an SW50.1 rotor (15 C) at 48,000 rpm, for 60 min in the case of A, and 85 min in the case of B. The ^{3}H counts represent DNA from the wild-type strain, whereas the ^{14}C counts represent DNA from the cured derivative.*

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